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Genome-based PCR primers for specific and sensitive detection and quantification of *Xylella fastidiosa*

Marta Francis[1], Hong Lin[2], Juan Cabrera-La Rosa[3], Harshavardhan Doddapaneni[4], and Edwin L. Civerolo[2,*]
[1]*Department of Plant Pathology, University of California Davis, Davis, CA, 95616, USA;* [2]*USDA-ARS, San Joaquin Valley Agricultural Sciences Center, 9611 So. Riverbend Ave., Parlier, CA, 93648, USA*
[3]*Department of Entomology, University of California, Riverside, CA, 92521, USA;* [4]*Department of Viticulture and Enology, University of California Davis, Davis, CA, 95616, USA; *Author for Correspondence (Phone: +1-559-596-2702; Fax: +1-559-596-2705; E-mail: eciverolo@fresno.ars.usda.gov)*

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Abstract

Xylella fastidiosa is an important pathogen of many commercial crops. Detection of *X. fastidiosa* is difficult due to low concentrations of the bacteria in insects and asymptomatic plant tissue, and non-uniform distribution in infected plants. A dual purpose conventional PCR and quantitative PCR (TaqMan™) system was developed for the generic detection of *X. fastidiosa* strains. Primers HL5 and HL6, designed to amplify a unique region common to the sequenced genomes of four *Xylella* strains, amplified a 221 bp fragment from strains associated with Pierce's disease of grapes, almond leaf scorch, and oleander leaf scorch disease and from DNA from an Xf strain associated with citrus variegated chlorosis. Standard curves were obtained using concentrations of *Xylella* ranging from 5 to 10^5 cells per reaction in water and grape extracts and 10–10^5 cells in insect DNA. Regression curves were similar, with correlation coefficients of $r^2 > 0.97$. In quantitative PCR, C_t values ranged between 20 and 36 cycles for 5–10^5 bacterial cells per reaction. No amplicons were obtained with several non-Xf bacterial strains tested including related plant pathogenic, grape endophytic bacteria and endosymbiotic bacteria isolated from glassy-winged sharpshooters. The method was evaluated for clinical diagnosis of Xf in grapes, almonds and insect vectors. The procedure described is reliable for detection of the pathogen with a high degree of sensitivity and specificity.

Abbreviations: ALSD – almond leaf scorch disease; CTAB – hexadecyltrimethylammonium bromide; GWSS – glassy-winged sharpshooter; ITS – internal transcribed spacer; OLSD – oleander leaf scorch disease; PCR – polymerase chain reaction; PD – Pierce's disease; Q-PCR – quantitative PCR; Xf – *Xylella fastidiosa*.

Introduction

Xylella fastidiosa (Xf) is an important plant pathogen that causes economic losses in agronomic and horticultural crops in North and South America, including grape, citrus, coffee, peach, almond, plum, alfalfa, as well as in several landscape trees and ornamental plants, such as elm, maple, mulberry, oak, sycamore, and oleander (Hopkins and Purcell, 2002).

The bacterium is limited to the xylem of infected hosts and is transmitted by several xylem-sap

feeding insect vectors (Purcell and Hopkins, 1996). With the recent introduction and spread of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* Say (Hemiptera: Cicadellidae) into California, there is the potential for increased incidence of Pierce's disease (PD) and almond leaf scorch disease (ALSD), as well as xylella diseases of other horticultural crops (Almeida and Purcell, 2003).

Reliable detection of Xf is essential for monitoring the spread of the pathogen. This has been achieved by isolation, serology (e.g., ELISA) and several PCR methods using primers designed for DNA fragments obtained by incubation of total Xf genomic DNA with restriction enzymes, random amplification (Minsavage et al., 1994; Pooler and Hartung, 1995), and conserved genes like 16S rRNA (Chen et al., 2000) and gyrB (Rodrigues et al., 2003).

A quantitative PCR procedure was developed for the diagnosis of PD, using conserved sequences of the 16S rRNA and 16S–23S internal transcriber spacers (ITS) (Schaad et al., 2002). Quantification of citrus variegated chlorosis (CVC) strains in Brazil was achieved (Oliveira et al., 2000) with primers based on the target sequence identified by random amplification of total genomic DNA (Pooler and Hartung, 1995).

With the availability of the sequences of the genomes of four Xf strains associated with CVC (strain 9a5c), PD (strain Temecula-1), ALSD (strain Dixon) and oleander leaf scorch disease (OLSD; strain Ann-1) (Simpson et al., 2000; Bhattacharyya et al., 2002; Van Sluys et al., 2003), unique Xf sequences can now be identified and compared with genomic sequences in closely related plant pathogenic bacteria (e.g., *Xanthomonas*) (da Silva et al., 2002) and several other microorganisms available in the data banks (http://www.ncbi.nlm.nih.gov).

Several protocols have been evaluated to overcome the effects of inhibitors in plant and insect extracts that interfere with the PCR reactions (Minsavage et al., 1994; Bextine and Miller, 2004). Other methods include immunocapture PCR (Pooler et al., 1997) and addition of Chelex 100 to avoid PCR inhibitors from insect vectors (Ciapina et al., 2004).

Detection of Xf in the GWSS using QPCR with SYBR Green was recently described (Bextine et al., 2005). However, SYBR Green is less specific and reliable than the TaqMan system because SYBR Green binds to any dsDNA, including the specific target DNA, nonspecific PCR products and primer dimers (Giulietti et al., 2001).

There is a need for standard PCR-based protocols for reliable clinical detection and identification of Xf and widely applicable for different crops and insect vectors. The overall goal of this work was to develop improved Xf detection and identification protocols (i.e., increased specificity and sensitivity and rapid and easy use) that include improved DNA extraction, and genome-wide based PCR primers and probes. The system was developed for application in quantitative PCR (Q-PCR) with the TaqMan system as well as conventional PCR (C-PCR). The protocol was evaluated for the detection and quantification of Xf in clinical plant (e.g., grape, almond) tissue, and in insect vectors (e.g., GWSS). However, the protocols can be used for analyses of other Xf host plants and Xf vectors.

Materials and methods

Bacterial strains

This study included 42 Xf strains associated with PD, ALSD and OLSD, and DNA from 10 Xf-CVC strains; 7 related plant pathogenic bacteria; 14 endophytic bacteria isolated from grapevines; and 5 endosymbiotic bacteria isolated from the GWSS in California (Table 1).

Grape, almond and insect vector samples

Samples were collected from vineyards with PD symptoms and almond from ALSD-affected orchards in different regions of California. Bacteria were initially isolated from petioles of infected grapes and almonds on PWG media (Hill and Purcell, 1995), and subsequently identified as Xf by PCR using primers RST31 /RST33 (Minsavage et al., 1994). Uninoculated and inoculated grapevines (*Vitis vinifera*) and almond (*Prunus amygdalus*) plants were used as sources of negative and positive plant tissue controls. Xylem fluid was collected from apparently uninfected grape cv. Thompson seedless in early spring (March). Dormant grapes were selected, branches were cut and bent until the fluid was released by natural pressure and collected in sterile plastic tubes.

Xf-free adult GWSS from greenhouse-reared colonies were used as negative insect controls.

Table 1. Specificity of primers HL5 and HL6 in quantitative PCR

Bacterial strains[a] (number) *Xylella fastidiosa*	Host[b]	Origin (source)[c]	QPCR (C_t)[d]
Reference strains			
PD Temecula	Grape	Temecula, CA (1)	28.6
ALS Dixon	Almond	Solano Co., CA (1)	28.5
OLS Ann-1	Oleander	Riverside, CA (1)	28.6
CVC 9a5c (DNA)[e]	Citrus	São Paulo, Brazil (2)	20.6
Other strains			
PD (9)	Grape	Kern, CA (3)	27.8
PD (3)	Grape	Riverside Co., CA (3)	29.4
PD (7)	Grape	Napa Co., CA (3)	28.6
PD (1)	Grape	Baja, Mexico (3)	27.8
ALS (1)	Almond	Tulare Co., CA (4)	28.4
ALS (1)	Almond	Contra Costa Co., CA (4)	28.3
ALS (3)	Almond	San Joaquin Valley, CA (4)	28.5
OLS (4)	Oleander	Riverside, CA (4)	28.6
JAB (9) (DNA)[e]	Citrus	Sao Paulo, Brazil (2)	20.6
Grape bacterial endophytes			
Pantoea (1)	Grape	Davis, CA (5)	–
Bacillus sp. (4)	Grape	Davis, CA (5)	–
Pseudomonas sp. (1)	Grape	Davis, CA (5)	–
Frigobacterium sp (1)	Grape	Davis, CA (5)	–
Burkhoderiales (1)	Grape	Davis, CA (5)	–
Erwinia sp (2)	Grape	Davis, CA (5)	–
Lactobacillus (2)	Grape	Davis, CA (5)	–
Staphylococcus sp (1)	Grape	Davis, CA (5)	–
Alcaligenes faecalis (1)	Grape	Davis, CA (5)	–
Plant pathogenic bacteria			
X.. campestris pv. campestris	Cabbage	Davis, CA (6)	–
X. axonopodis pv. vesicatoria	Tomato	Davis, CA (6)	–
X. arboricola pv. juglandis	Walnut	Davis, CA (6)	–
X. oryzae pv. oryzae	Rice	Davis, CA (7)	–
Xanthomonas fragariae	Strawberry	ATCC 33239 (8)	–
Agrobacterium vitis	Grape	California (5)	–
Agrobacterium tumefaciens	Grape	California (5)	–
Insect endosymbiotic bacteria			
Un-identified (5)	GWSS	S.J. Valley, CA. (3)	–

[a]Bacterial strains used in this study.
[b]Host from which the strain was originally isolated.
[c]Geographical origins and sources and origin of bacterial strains: (1) B. Kirkpatrick, University of California, Davis; (2) E. Lemos (Universidad de Sao Paulo, Brazil); (3) this study; (4) A.H. Purcell, University of California, Berkeley; (5) D. Darjean, University of California, Davis; (6) L. Bolkan, University of California, Davis; (7) P. Ronald, University of California, Davis; and (8) American Type Culture Collection.
[d]Q-PCR results with primer set HL5/HL6. Bacterial suspensions were adjusted to contain approx. 10^3 cells μl^{-1} per reaction. Xf-CVC DNA was used at 1 ng per reaction. Positive Q-PCR results were confirmed by visualization of bands following electrophoresis in agarose gels. Q-PCR, results are expressed as C_t values, defined as the PCR cycle number at which the fluorescent signal crossed the threshold. C_t values are the mean of three replications. C_t values > 37 were considered to be negative.
[e]DNA from Xf-CVC strains were used in this assay.

Xf-inoculative GWSS were obtained after exposure to Xf-infected almonds (3-day acquisition access period) and then transferred to periwinkle plants (*Catharanthus roseus*) (4-day inoculation access period). Periwinkle plants were assayed 8 weeks later by DAS-ELISA to confirm transmission of Xf. The insects associated with Xf transmission were used as positive controls in the PCR assays.

Adult GWSS insects were collected from a citrus orchard adjacent to a PD-affected grape vineyard in

the lower San Joaquin Valley of California. The insects were stored frozen at −80 °C until processed.

DNA extraction from plants and insects samples

The DNA extraction method for grape tissue (Lin and Walker, 1997) was modified for rapid and easy processing of grape, almond and insect samples using the FastPrep, Bio 101, Cell Disrupter (Qbiogene, Inc., Carlsbad, CA, USA). Petiole and midvein tissue (0.5–1.0 cm long) from three leaves (~200 mg) or whole insects were homogenized in a 2 ml tube with a ceramic bead in 1.5 ml of the extraction buffer (20 mM EDTA, 350 mM Sorbitol in 100 mM Tris–HCl, pH 7.5 plus 2.5% w/v PVP and 0.2% (v/v) of β-mercaptoethanol) as described previously (Lin and Walker, 1997). The tubes were centrifuged at $16{,}000 \times g$ for 20 min to collect Xf bacteria in the samples and the supernatant was discarded to remove as much soluble inhibitors as possible. The pellet containing homogenized plant tissue was resuspended in 300 μl of DNA resuspension buffer containing 20 mM EDTA, 350 mM Sorbitol in 100 mM Tris–HCl, pH 7.0, plus 300 μl of DNA lysis buffer (50 mM EDTA, 2 M NaCl, 2% (w/v) CTAB in 200 mM Tris–HCl, pH 7.5, and 200 μl of 5% sarcosyl), mixed well and incubated at 65 °C for 45 min (Lin and Walker, 1997). DNA purification was done in the same tube by adding 800 μl chloroform–octanol (24:1), and after centrifugation at $5000 \times g$ for 10 min the supernatant was transferred to a new tube for a second chloroform–octanol extraction. Nucleic acid was precipitated with an equal volume of isopropanol for 30 min at −20 °C, centrifuged at $12{,}000 \times g$ for 10 min, and the pellet rinsed twice with 70% ethanol. The total DNA preparation was resuspended in 200 μl of 0.5X TE.

Design of primers and probe for generic detection of Xylella fastidiosa *strains*

A specific region common to the four published genomes (Simpson et al., 2000; Bhattacharyya et al., 2002; Van Sluys et al., 2003) of Xf strains (Temecula-1, Dixon, Ann-1, CVC 9a-5c) was identified based on *in silico* genome analysis. The genome sequence of Xf strain Temecula-1 (AE 009442) was used as reference sequence for primer design. Multiple primer pairs with similar design parameters (GC = 50%, T_m = 58 °C, primer length ~20 bp, and self dimer/cross dimer $\Delta G \geq -5$ kcal mol^{-1} and amplicon sizes ranging from 150 to 300 were selected for the PCR target region. The Primer Premier 5 software (Premier-Biosoft, Palo Alto, CA) was used for primer design. After initial screening, the primer pair HL5 forward (5′-AAGGCAATAAACGCGCACTA-3′) and HL6 reverse (5′-GGTTTTGCTGACTGG CAACA-3′), was selected for further protocol optimization. PCR products obtained from grapes, almond and GWSS samples were used for forward and reverse direct sequence using ABI PRISM® BigDye® Terminator sequencing kit (Biosystem™ Foster City, CA).

The sequence specificity of each amplicon was checked *in silico* by BLAST analysis against all available microbial genome sequence databases (*E*-value e^{-30} to e^{-5}) in GenBank to verify that the sequences were unique to Xf, no significant match was found.

Conventional PCR (C-PCR)

Bacterial suspensions were adjusted to 0.2 $A_{600\ nm}$ (containing approx. 2×10^8 Xf cells per ml), and serially diluted in sterile distilled water. Grape and almond DNA preparations were diluted in 0.5X TE buffer to a concentration equivalent to 1 mg fresh tissue per μl. Total GWSS DNA per insect was resuspended in 100 μl 0.5X TE buffer and diluted 10^{-1} in sterile distilled water.

To analyze the effect of inhibitors on PCR, aliquots of DNA from healthy grape tissue or non-inoculative insects was mixed with the same volume of bacterial suspension and used in comparative assays to determine the level of Xf detection sensitivity.

One microliter of sample was added to 24 μl of master mix containing 2.5 μl of 10X PCR HotStart Master Mix, 0.5 μl of dNTPs (10 mM), 0.2 μM of each primer (HL5/HL6), 1 unit of HotStart Taq Polymerase, (Qiagen Inc., Valencia, CA, USA) with 1.5 mM $MgCl_2$. Amplification was performed in 9700 ABI thermocycler, (Applied Biosystem, Foster City, CA, USA). PCR cycling parameters were: 95 °C for 15 min, followed by 40 cycles (95 °C for 10 s, 60 °C for 15 s and 72 °C for 30 s). Aliquots (10 μl) of the PCR reaction were electrophoresed on a 2% agarose gel (1X TAE buffer), stained with ethidium bromide and observed under UV light. C-PCR using primers RST31/RST33 was performed according to the protocol described by Minsavage et al. (1994).

Quantitative PCR (Q-PCR)

Q-PCR was performed in 25 μl tubes in a Smart Cycler (Cepheid, Sunnyvale, CA, USA), with 4.5 mM $MgCl_2$, 0.2 μM of each primer (HL5/HL6), 0.4 μM Probe (5′TGGCAGGCAGCAACGATACGGCT3′) labelled with FAM (6-carboxyfluorescein) at the 5′ end, and BHQ1™ at the 3′ end as a non-fluorescent quencher (Sigma Genosys, Woodland, TX, USA), and 1 unit of HotStart Taq Polymerase. The amplification protocol was 95 °C for 15 min and 40 cycles, 95 °C for 10 s, 60 °C for 15 s (optics on) and extension at 72 °C for 30 s. The threshold was established at 10 units of fluorescence over the background subtraction according to the Smart Cycler Software instructions (Cepheid, Sunnyvale, CA, USA) and results were recorded as the cycle threshold (C_t).

Standard curves

Standard curves relating the amount of fluorescence to Xf cells per reaction were obtained using a series of 10-fold dilutions of bacteria using diluents that included xylem fluid and DNA extracted from healthy grape tissue to mimic the composition of xylem fluid or extracts of infected grape tissue. Similarly, Xf-free adult GWSS extracts were amended with 5–10^5 Xf cells per μl. Serial dilutions of genomic Xf DNA in water were also assayed at concentrations of 1 pg–100 ng per μl.

Serology

Petioles from grape and almond samples were assayed by DAS-ELISA using a commercial kit for Xf diagnosis according to the manufacturer's instructions (AGDIA, Elkhart, IN, USA). Mean $A_{492\ nm}$ values from duplicate wells that exceeded the background from extracts of uninoculated controls by three standard deviations were considered positive.

Results

DNA extraction

The DNA procedure described here yielded higher quality of DNA for PCR than previously described methods for Xf detection (Minsavage et al., 1994; Pooler et al., 1997; Bextine and Miller, 2004; Bextine et al., 2004 and Ciapina et al., 2004). The quality and amount of DNA obtained were verified by gel electrophoresis and ethidium stained gels (data not shown). There were no inhibitory effects of the PCR reactions because most of the polyphenolic and polysaccharide compounds were removed at the beginning of the extraction procedure when the supernatant was discarded. The total sample is recovered in one single tube resulting in efficient recovery of Xf DNA from plant and insect tissue samples with low amounts of the bacteria. Undiluted DNA from early season grape and almond samples was suitable for PCR directly. When high concentrations of inhibitors were present late in the season, best results were obtained with plant DNA-containing extracts diluted 10^{-1}. DNA-containing preparations from GWSS tissue were most suitable for PCR when diluted 10^{-1}.

General detection of PD, ALSD, OLSD and CVC Xf strains

Primer HL5/HL6 amplified a 221 bp product (Figure 1) from Xf strains associated with PD (21), ALSD (6), and OLSD (5), as well as from DNA from CVC strains (10). The specificity of primers HL5/HL6 was assessed by Q-PCR using bacterial concentrations of 10^3 cells μl^{-1} reaction with different Xf strains (PD, OLSD and ALSD). C_t values ranged from 28 to 29 (Table 1). In Q-PCR, the C_t using 1 ng Xf-CVC DNA μl^{-1} per reaction was 20. This is equivalent to 10^5 Xf cells μl^{-1} per reaction. The C_t values for non-Xf bacteria (grape endophytes, insect endosymbionts and selected plant pathogenic bacteria) at the same concentration were >37 (Table 1). Therefore, we conclude that the primer locus we selected here is specific to *Xylella* based on the available genome sequence information in GenBank. Moreover, C_t values >37 were considered negative in Q-PCR analyses for Xf.

Sequence validation

PCR amplicons produced by primer set HL5/HL6 from infected grape, almond and GWSS were sequenced to confirm sequence identity. BLAST analyses of the amplicon sequences against the genomes of *Xylella* and related genera of plant

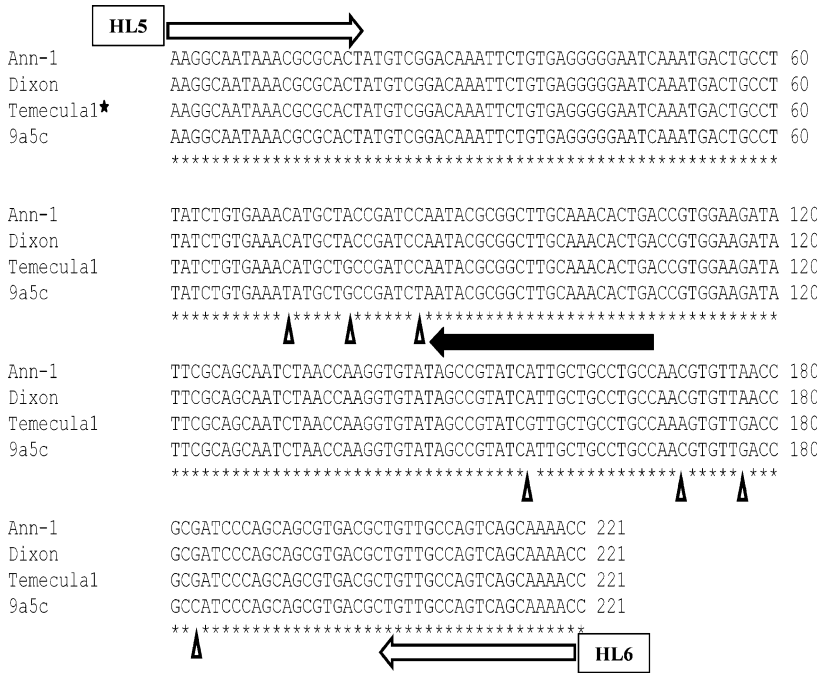

Figure 1. Region in the *Xylella fastidiosa* (Xf) genome amplified with primers HL5/HL6. Alignments of the amplified region from the genomes of the four reference Xf strains (Ann-1, Dixon, Temecula-1, and 9a5c) used in this study. White arrows indicate the positions and orientations of the primers HL5 (forward) and HL6 (reverse). Black arrow indicates the position and orientation of the fluorescence probe used for real-time PCR assays. Triangles indicate single nucleotide polymorphisms compared with the Temecula-1 strain (*) used as a reference strain in this study.

pathogenic bacteria in GenBank only matched sequences in the genomes of the four Xf strains. To further test sequence specificity, *in silico* BLAST analyses were performed using the full length amplicon sequence against ~389 microbial sequences in GenBank. No significant match was reported. Furthermore, we used the HL5 and HL6 primer sequences (~20 bp) to search for short, nearly exact BLAST matches against all organisms. In no case did both primers hit the same organisms with significant matches.

Sensitivity

Standard curves of Xf diluted in water, grape DNA and insect DNA were similar ($y = 10.3–0.26x$, $r^2 > 0.97$). The limit of detection was 5 Xf cells per reaction in water and in grape DNA solution ($C_t = 36$), and 10 Xf cells in insect DNA solution ($C_t = 37$). A strong inhibitory effect was observed with undiluted xylem fluid. The limit of Xf detection in undiluted xylem fluid was 10^3 cells per reaction ($C_t = 34.2$; Table 2).

When Xf DNA was diluted in water, C_t values ranged from 13–33 for 100 ng μl^{-1} to 1 pg μl^{-1}, respectively. The C_t value for 1 ng of Xf DNA was 20.3 and equivalent to that obtained with 10^5 Xf cells per reaction ($C_t = 20$). This agrees with the copy number expected for 1 ng of genomic dsDNA of the size of the Xf-PD genome.

The comparative sensitivity of primers HL5/HL6 and RST31/RST33 was tested in C-PCR using the same DNA extracts. Comparable results were obtained with both primer sets when bacterial suspensions were diluted in water. However, when bacterial suspensions were mixed with DNA extracted from grape or insects, the sensitivity of Xf detection by PCR using primer set RST31/RST33 was reduced 100-fold compared to primer set HL5/HL6 (Table 2).

Detection and quantification of Xf in clinical samples

The unique 221 bp amplicon produced by primers HL5/HL6 was obtained from clinical samples

Table 2. Comparative sensitivity of detection of *Xylella fastidiosa* Temecula-1 strain by quantitative PCR and conventional PCR with primers HL5 and HL6 and previously described set RST31 and RST33

Bacterial concentration	HL5/HL6							RST31/RST33		
	Q-PCR C_t[b]				C-PCR[c]			C-PCR		
Xf cells per reaction[a]	W	G	I	GF	W	G	I	W	G	I
10^5	19.9	20.1	21.7	29.8	+	+	+	+	+	+
10^4	23.9	23.2	25.3	32.3	+	+	+	+	+	+
10^3	28.8	28.2	31.2	34.2	+	+	+	+	+	+
10^2	30.7	32.3	35.9	–	+	+	+	+	$+^w$	$+^w$
10^1	34.4	35.7	36.9	–	+	+	+	+	–	–
5	36.0	36.6	–	–	+	$+^w$	$+^w$	+	–	–
2	–	–	–	–	–	–	–	–	–	–
0	–	–	–	–	–	–	–	–	–	–

[a]Xf cell suspensions were diluted in water (W), grapevine leaf petiole tissue DNA extracts (G), insect tissue DNA extracts (I), or grapevine xylem fluid (GF). One μl of suspension was used in each reaction.
[b]Values represent the mean of three replications. $C_t > 37$ considered to be negative (–).
[c]Amplicons observed in agarose gels: (+) positive, ($+^w$) weakly positive and (–) negative.

(grapevines, almonds and GWSS) containing Xf (Figure 2A-Lanes 2, 4 and 6). No amplification was obtained with healthy grape, almond or GWSS tissue extracts (Figure 2A-Lanes 1, 3 and 5). Reference strains Xf-PD (Temecula 1) and Xf-ALSD (Dixon) produced identical size amplicons (Figure 2A-Lanes 7 and 8). In Q-PCR analyses, grape and almond samples had higher concentrations of Xf (C_t = 20), than reactions containing 10^4 cells of reference strains (C_t = 24). Lower bacterial levels were found in GWSS samples by C-PCR (Figure 2A, Lane 6) and Q-PCR (C_t = 35; Figure 2B, Lane 6).

Of 28 grape and 38 almond samples assayed, 14 (50.0%) grape and 27 (71.0%) almond samples were positive with primer set HL5/HL6 by C-PCR and Q-PCR (Table 3). The C_t values of field collected Red Globe samples were lower (C_t = 24) than those for Cabernet Sauvignon samples (C_t = 34). The C-PCR system using primers HL5/HL6 described herein was able to detect the bacteria in asymptomatic almond shoots in early spring (May). However, Xf was not detected in the same samples using primers RST31/RST33. In late summer (September) when characteristic leaf scorch symptoms were well developed, and the pathogen was detected by ELISA, the results obtained in C-PCR with both sets of primers (HL5/HL6 and RST31/RST33) were similar. The levels of Xf detected in almond samples late in the season (October) were estimated to range from 10^5 cells mg^{-1} of tissue (cv. Sonora C_t = 21) to 10^3 cells mg^{-1} of tissue (cv. Price C_t = 29) (Table 3).

The highest C_t values (33–34) were obtained with GWSS extractions from transmission assays and field collected insects. The amount of bacteria per sample (equivalent to 10^{-1} dilution of 100 μl total DNA extracted per whole insect), was estimated to be between 10 and 100 cells. These low levels were detected by C-PCR with primers HL5/HL6, but not with primers RST31/RST33 (Table 2). This concentration is also below the limit of detection by ELISA.

Discussion

The DNA extraction method for grape rootstocks (Lin and Walker, 1997), modified for small sample preparations as described here, minimized interference by PCR inhibitors present in plant and insects. The use of a single microfuge tube for tissue homogenizing and total DNA extraction permits efficient recovery of target DNA in samples with low amounts of Xf bacteria, such as in plant tissue samples in early spring and in insects. In addition, this facilitates accurate quantification of Xf present in each sample. The primers HL5 and HL6 are highly specific and sensitive for Xf detection by C-PCR and Q-PCR because they amplify a sequence that is common to the completely sequenced genomes of four Xf strains but

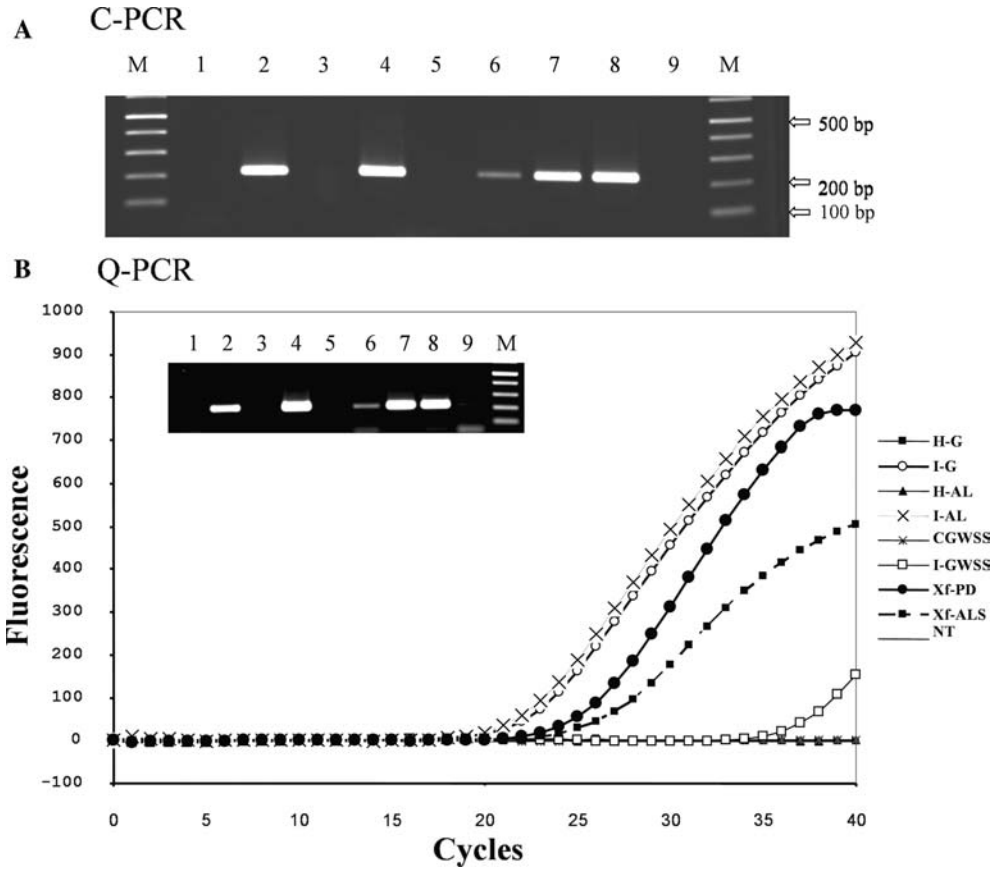

Figure 2. Conventional and quantitative PCR analyses of field-collected plant and insect samples using primers HL5 and HL6. A, Amplicons obtained by conventional PCR (C-PCR) with tissue extracts from Xf infected grapevines and almond trees, and Xf-inoculative GWSS samples (lanes 2, 4 and 6), reference Xf strains PD-Temecula 1 (lane 7) and ALSD-Dixon (lane 8). No amplicons were obtained with similar extracts from healthy grapevine and almond tree samples, non-inoculative GWSS, and no template control (lanes: 1,3,5 and 9). *M* = 100 bp molecular weight ladder. B, Quantitative PCR (Q-PCR) with the same samples as in A. Axes: *y* = cumulative fluorescence, *x* = cycle threshold (C_t). Tissue extracts from field-collected samples of grapevines and almond trees (lanes 2 and 4) have similar C_t values (C_t = 20) as the reference strains at 10^4 Xf cells per reaction (PD and ALSD C_t = 24). Xf-inoculative GWSS extracts had the highest C_t (C_t = 35). No signal was obtained with healthy tissue extracts.

unknown in other bacteria based on the available GenBank information. The Xf genome contains a single copy of the target region making it ideal for estimation of the bacterial concentration by Q-PCR. The size of the HL5/HL6 amplicon (221 bp) allows greater specificity and sensitivity in clinical detection and identification of Xf by C-PCR than was achieved with primer set RST31/RST33 (Minsavage et al., 1994).

The 42 tested Xf isolates (including strains associated with PD, ALSD and OLSD), as well as genomic DNA from 10 Xf strains associated with CVC, all produced only the 221 bp product. No amplification product was obtained from seven strains of closely related plant pathogenic bacteria, 14 strains of endophytic bacteria isolated from grapevines, or five strains of bacterial endosymbionts isolated from insects.

Xf insect vectors generally harbour low concentrations of the pathogen. Hill and Purcell (1995) estimated that less than 100 live bacteria within an insect vector were sufficient for the transmission of Xf to grape plants. This population is below the detection limit of isolation *in vitro* and ELISA and explains the reported requirement for nested PCR to detect Xf in insect vectors (Pooler et al., 1997; Rodrigues et al., 2003; Ciapina et al., 2004). Xf was readily detected by PCR using primers HL5 and HL6 in grapevines and almond trees (early in the spring), as well as in

Table 3. Clinical detection of *Xylella fastidiosa* in field collected grapes, almonds and glassy-winged sharpshooter

Sample	Number of samples				Quantification	
	Total	Symptoms PD or ALS	ELISA (+)	C-PCR (+)	Q-PCR (C_t)[d]	Xf cells[e]
Grape						
Thompson seedling	5	0	0	0	–	0
Cabernet Sauvignon	5	0	0	0	–	0
Chardonnay inoculated[a]	4	4	4	4	19.1 ± 1.2	$>10^5$
Cabernet Sauvignon	8	5	5[w]	5	33.8 ± 2.7	$<10^2$
Red Globe	6	4	4	4	23.8 ± 5.9	10^3–10^4
Almond						
Almond healthy	4	0	0	0	–	0
Price (Fresno)	6	5	5	5	28.9 ± 5.1	10^2–10^3
Sonora (Fresno)	5	3	3	3	20.6 ± 1.7	10^4–10^5
Sonora (Bakersfield)	8	6	6	6	28.6 ± 1.3	10^2–10^3
Sonora (May)[b]	5	0	0	5	29.3 ± 2.5	10^2–10^3
Sonora (July)[b]	5	5	5[w]	4	29.7 ± 4.5	10^2–10^3
Sonora (Sept.)[b]	5	5	5	4	29.1 ± 1.2	10^2–10^3
GWSS						
Control (–)	10	n/a	–	0	–	0
Trans. assays[c]	46	n/a	+	46	34.4 ± 0.5	10–10^2
Field samples	80	n/a	n/a	24	32.8 ± 0.4	10–10^2

[a]Tissue from Chardonnay grapevine inoculated with the Temecula-1 strain was used as positive control.
[b]Set of selected almond trees determined to be infected with Xf based on presence of ALSD symptoms, ELISA and isolation of the pathogen in the previous year.
[c]Positive GWSS were verified by transmission of Xf to periwinkle plants (ELISA positive).
[d]Mean C_t values of three replicates for each Xf concentration.
[e]The number of Xf cells estimated per mg of plant tissue or μl of insect tissue estimated with standard curves.

insect vectors, without the need for nested PCR. For the clinical almond samples, the identification of infected and uninfected individuals was confirmed later in the season by ELISA and pathogen isolation. Inoculative insects containing Xf bacteria were confirmed by pathogen transmission.

In the real-time system developed by Schaad et al. (2002), the 16S RNA primers were more sensitive than the ITS primers due to the high number of copies of template DNA per Xf cell. However, in order to achieve increased sensitivity and specificity, they recommended using both primer sets. Further, the amplicon was smaller than 80 bp, making it difficult to distinguish it from primer dimers in agarose gels. The protocol described here overcomes both of the above shortcomings. Moreover, the TaqMan system used here is more specific than the SYBR Green method (Giulietti et al., 2001; Bextine et al., 2005).

The DNA extraction and Xf detection protocols described here permit the recovery of the entire sample into one tube and is suitable for detecting of low levels (e.g., 5–10 cells per reaction) of the pathogen in clinical samples. Currently, these methods are being applied to the epidemiological analyses of Xf and xylella diseases in grapevines, almonds and insect vectors in the San Joaquin Valley of Central California. The procedures described herein are appropriate for Xf detection and identification when a high degree of sensitivity is required, such as disease surveys, and plant quarantine and certification programmes. These methods could also be used for quantification of Xf in breeding programmes to assess relative susceptibility or resistance to Xf infection.

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